Annu. Rev. Plant Biol. 2002. 53:449–75
DOI: 10.1146/annurev.arplant.53.100301.135233
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RUBISCO: Structure, Regulatory Interactions, and Possibilities for a Better Enzyme

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Key Words carbon dioxide, catalysis, chloroplast, photosynthesis, protein structure

■ **Abstract** Ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (Rubisco) catalyzes the first step in net photosynthetic CO_2 assimilation and photorespiratory carbon oxidation. The enzyme is notoriously inefficient as a catalyst for the carboxylation of RuBP and is subject to competitive inhibition by O_2, inactivation by loss of carbamylation, and dead-end inhibition by RuBP. These inadequacies make Rubisco rate limiting for photosynthesis and an obvious target for increasing agricultural productivity. Resolution of X-ray crystal structures and detailed analysis of divergent, mutant, and hybrid enzymes have increased our insight into the structure/function relationships of Rubisco. The interactions and associations relatively far from the Rubisco active site, including regulatory interactions with Rubisco activase, may present new approaches and strategies for understanding and ultimately improving this complex enzyme.

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INTRODUCTION

As the entry point of CO_2 into the biosphere, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is central to life on earth. Its very slow catalytic rate of a few per second, the low affinity for atmospheric CO_2, and the use of O_2 as an alternative substrate for the competing process of photorespiration together make Rubisco notoriously inefficient as the initial CO_2-fixing enzyme of photosynthesis. Consequently, land plants must allocate as much as 50% of their leaf nitrogen to Rubisco, making this single enzyme the most abundant protein in the world (35).

As the rate-limiting step of photosynthesis in both C_3 (55) and C_4 plants (144), Rubisco is often viewed as a potential target for genetic manipulation to improve plant yield (83, 126, 132). The food, fiber, and fuel needs of an ever-increasing human population and shortages in the availability of water for agriculture are challenges of the twenty-first century that would be impacted positively by successful manipulation of Rubisco in crop plants. During the past 10 years, resolution of a variety of Rubisco atomic structures has increased our understanding of the reaction mechanism of the enzyme. Based on this information, the new conventional wisdom is that improving Rubisco will not be simple but will require multiple mutations that subtly change the positioning of critical residues within the active site. Considering the explosion of new knowledge that has occurred in just the past few years regarding the interactions and associations that impact the structure, function, and regulation of Rubisco, there is reason to believe that successful manipulation of Rubisco may yet be achieved.

GENERAL STRUCTURAL AND FUNCTIONAL CONSIDERATIONS

Wealth of Rubisco Structures and Sequences

Besides being one of the slowest, Rubisco is also one of the largest enzymes in nature, with a molecular mass of 560 kDa. In land plants and green algae, the chloroplast *rbcL* gene encodes the 55-kDa large subunit, whereas a family of *rbcS* nuclear genes encodes nearly identical 15-kD small subunits (reviewed in 28, 124). Following posttranslational processing of both subunits, small subunits are added to a core of chaperone-assembled large subunits in the chloroplast (reviewed in 108, 126). The resulting Form I Rubisco holoenzyme is composed of eight large and eight small subunits (Figure 1*A*). Variations on this theme

include the Form II Rubisco of some prokaryotes and dinoflagellates consisting of a dimer of only large subunits (89, 132, 151) and the Form I Rubisco of nongreen algae produced from *rbcL* and *rbcS* genes that are both chloroplast encoded (132). In still another variation, the Rubisco from archaebacteria is neither Form I or II, but a decamer comprising five large-subunit dimers (82).

More than 20 Rubisco X-ray crystal structures now exist within the Protein Data Bank (10). These range from the homodimeric holoenzyme of *Rhodospirillum rubrum* (4) that provided the first glimpse of the active site to the high-resolution structures of spinach Rubisco with bound substrate, product, and transition-state analogs (3, 68, 133–136). The C-terminal domain of the large subunit of every Rubisco enzyme forms a classic α/β-barrel. Residues predominately in the loops between β strands and α helices interact with the transition-state analog 2-carboxyarabinitol 1,5-bisphosphate (CABP) (Figure 1*B*). Because several N-terminal-domain residues of a neighboring large subunit also participate as designated "active-site" residues (Figure 1*C*), the functional unit structure of Rubisco is a large-subunit dimer (Figure 1*A*). Four pairs of associated large subunits are capped on each end by four small subunits, each of which interacts with three large subunits (Figure 1*A*). Because large subunits of Form II enzymes contain all the structural elements required for catalysis, the origin and role of the small subunit in Form I enzymes remain enigmatic.

More than 2000 *rbcL* and 300 *rbcS* sequences now reside within GenBank, generated primarily for phylogenetic reconstructions (21, 22, 63). The deduced large-subunit sequences are fairly conserved, and any differences in length occur primarily at the N and C termini. (Throughout this review, numbering of large-subunit residues will be based on the sequence of the spinach large subunit.) *rbcL*-like sequences have also been found in prokaryotes that do not possess photoautotrophy via the Calvin cycle (reviewed in 132). However, the deduced products of these genes appear to lack certain residues essential for carboxylation, indicating that they may have other functions in the cell (48).

Small subunits are more divergent than large subunits. (Throughout this review, numbering of small-subunit residues will be based on species-specific sequences.) Whereas land-plant and green-algal small subunits generally have larger loops between β strands A and B, some prokaryotes and all nongreen algae have longer C-terminal extensions. Small but significant regions of sequence identity have been observed between the *Synechococcus* small subunit and a protein associated with carboxysome assembly (98), indicating that there may be a shared interaction with the Rubisco large subunit (reviewed in 62) or divergence from a common ancestral protein of unknown function.

Catalytic Mechanisms and Kinetic Insights

FIRST PARTIAL REACTION Much is known about the Rubisco catalytic mechanisms from chemical modification, directed mutagenesis, and structural studies (reviewed in 23, 53, 124). Carbamylated Rubisco with bound Mg^{2+} (see below)

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binds RuBP and converts it to the 2,3-enediol(ate) form (enol-RuBP). This first partial reaction requires that a proton be abstracted from the C-3 of RuBP (66). Whereas some controversy surrounded the nature of the requisite base (68, 79), it is now thought that the free oxygen atom of the activator carbamate on Lys-201 is in a suitable environment to accept this proton (3, 23, 91). The basicity of the free carboxylate oxygen appears to be influenced by Asp-203 and Glu-204, two residues that together with carbamylated Lys-201 ligate the Mg^{2+} (Figure 1*C*). These residues are essential for catalysis (45), but they have not yet been altered in ways to directly test their involvement in proton abstraction. Once abstracted from RuBP, the proton is then shuttled in turn to the oxygen atom at C-2 of enol-RuBP, ε-amino group of Lys-175, and then to 3-phosphoglycerate (50, 51).

CONFORMATIONAL CHANGES Carbamylation of Lys-201 occurs spontaneously at slightly alkaline pH (80) and is stabilized by Mg^{2+} and various active-site residues (reviewed in 23). Carbamylation is required to "activate" the enzyme, converting it from a catalytically incompetent to a catalytically competent form. The participation of the carbamate and Mg^{2+} in the catalytic mechanism constrains the ways in which the active site can be modified to impact specificity or turnover (16, 49, 53).

Carbamylation causes only minor changes in the conformation of the Rubisco large subunit, primarily affecting residues in the loop between β strands B and C of the N-terminal domain (118, 133). In contrast, binding of RuBP and other phosphorylated ligands induces several major structural changes in and around the active site (91, 118, 133, 136). Most obvious is a 12-Å shift of α/β-barrel loop 6 from a retracted (open) to an extended (closed) position (118, 133) (Figure 1*B*). In the extended position, Lys-334 of loop 6 interacts with the C-1 phosphate (P1) of the bound ligand, as well as with Glu-60 at the C-terminal end of β strand B of a neighboring large subunit (Figure 1*C*). Other changes include rotation of the N-terminal domain, which positions the βB-βC loop over the bound substrate, and extension of the C-terminus across the face of the subunit, which may stabilize loop 6 in the extended conformation via ionic interactions (3, 68) (Figure 2). These interactions involve Asp-473, a residue that has been proposed to be a latch site for holding the loops in the extended positions (32). The extended loops in the closed conformation shield the active site from solvent.

SECOND PARTIAL REACTION AND CO_2/O_2 SPECIFICITY It is the second, irreversible partial reaction, the addition of gaseous CO_2 or O_2 to the enol-RuBP, that determines the specificity and rate of the overall reaction (15, 23, 95). The closure of loop 6 affects the position of the ε-amino group of Lys-334 (Figure 1*C*) that, along with Mg^{2+}, stabilizes quite similar transition states arising from the reaction of either CO_2 or O_2 with enol-RuBP (16, 23). The resulting products, 2-carboxy-3-ketoarabinitol 1,5-bisphosphate or 2-peroxy-3-ketoarabinitol 1,5-bisphosphate (12, 52, 95), are then protonated and hydrated to produce two molecules of 3-phosphoglycerate or one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate, respectively.

The preference of Rubisco for CO_2 versus O_2 is represented by the specificity factor (Ω), the ratio of the catalytic efficiency (V_{max}/K_m) of carboxylation (V_c/K_c) to that of oxygenation (V_o/K_o). Ω, which determines the ratio of the velocity of carboxylation (v_c) to that of oxygenation (v_o) at any specified concentrations of CO_2 and O_2 (57, 70), can be expressed as

$$\Omega = V_cK_o/V_oK_c = v_c/v_o \times [O_2]/[CO_2]. \quad (1)$$

Because Ω is determined by the second, rate-limiting partial reaction (95), one can further define this relationship based on transition-state theory as

$$\ln \Omega = \ln k_c/k_o = \left(\Delta G_o^{\ddagger} - \Delta G_c^{\ddagger}\right)/RT, \quad (2)$$

where k_c and k_o are the rate constants for the competing partial reactions, $\Delta G_c^{\ddagger}$ and $\Delta G_o^{\ddagger}$ are the carboxylation and oxygenation free energies of activation, R is the gas constant, and T is the absolute temperature (15, 16). Because $\Delta G_o^{\ddagger}$ is greater than $\Delta G_c^{\ddagger}$ (and k_o is smaller than k_c), k_o increases faster with temperature than does k_c (16). This accounts for observed decreases in Ω as temperature increases (15, 58, 139). For Rubisco enzymes from organisms as divergent as *R. rubrum* ($\Omega = 15$) and spinach ($\Omega = 80$), $\Delta G_o^{\ddagger} - \Delta G_c^{\ddagger}$ differs by less than 6 kJ mol^{-1} (16). Because this is less energy than that of a single hydrogen bond, rather subtle changes in protein structure are likely responsible for substantial changes in the gaseous substrate specificity of Rubisco.

Defining a Better Enzyme

Although Ω receives much attention when discussions turn toward the quest for a better enzyme (83), Ω does not provide a direct measure of the rates, rate constants, or catalytic efficiencies of either carboxylation or oxygenation. It represents the difference between the free energies of activation for the oxygenation and carboxylation transition states (Equation 2). Ω is a constant for each Rubisco enzyme, equal to the ratio of carboxylation to oxygenation catalytic efficiencies at a given temperature (Equation 1). As a ratio, Ω does not measure how fast the enzyme fixes CO_2 at any given concentrations of CO_2 and O_2. Consequently, to determine if a Rubisco enzyme is truly "better," one needs to measure the amount of CO_2 fixed minus the amount potentially lost via photorespiration. Laing et al. (70) used Rubisco kinetics to define net photosynthesis (Pn) as

$$Pn = v_c - tv_o = (V_cK_o([CO_2] - [O_2]/\Omega))/(K_o[CO_2] + K_cK_o + K_c[O_2]), \quad (3)$$

where t equals 0.5, i.e., the moles of CO_2 lost per mole of O_2 fixed (92). Thus, it is useless to replace a low-Ω enzyme with a high-Ω enzyme (83) if the individual kinetic constants indicate that Pn will not increase at the CO_2 and O_2 concentrations that occur in vivo.

DIVERGENCE OF KINETIC PROPERTIES Ω values differ between Rubisco enzymes from divergent species (57). Form II enzymes of certain prokaryotes and dinoflagellates have the lowest values ($\Omega = 15$) (57, 151), whereas Form I enzymes from

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nongreen, eukaryotic algae have the highest ($\Omega = 100$–240) (101, 102, 139). The Ω values of the less-divergent Form I enzymes of eubacteria, e.g., cyanobacteria ($\Omega = 40$), green algae ($\Omega = 60$), and land plants ($\Omega = 80$–100), fall between these two extremes (57, 126). Because there is an inverse correlation of Ω with V_c (57, 102, 139), it is difficult to tell if one enzyme is better than another in vivo. For example, the Form II Rubisco of *R. rubrum*, an obligate anaerobe for photosynthetic CO_2 fixation, has a very low Ω value, but a high V_c (57). The low Ω value is inconsequential in this microorganism because anaerobic growth coupled with the high V_c ensures a high v_c in vivo. Cyanobacteria, green algae, and C_4 plants have CO_2-concentrating mechanisms (reviewed in 62, 85, 123), reducing the importance of Ω and K_c in vivo. Although the thermophilic red alga *Galdieria partita* has the highest Ω value yet reported for a Rubisco enzyme assayed at 25°C ($\Omega = 240$) (139), this value falls below 80 (and its k_c and k_o both increase) when the enzyme is assayed at 45°C, the temperature at which *Galdieria* is normally cultured. Because the Rubisco enzymes of C_3 plants have evolved under conditions of ambient temperature and high O_2 concentration, one is tempted to conclude that these must be the "best" Rubisco enzymes. However, if transferred to a cyanobacterium, which has a CO_2-concentrating mechanism (reviewed in 62), the relatively low V_c values of the land-plant enzymes would result in a decrease in Pn, even though the Ω value has doubled.

SELECTION FOR A BETTER ENZYME Attempts have been made to directly select for "better" Rubisco enzymes in vivo under CO_2-limited conditions (29, 96, 131; reviewed in 124, 125). However, one cannot culture enough single-celled organisms to select for more than a few substitutions simultaneously (124). Clearly, if a single amino-acid substitution could increase Pn, it would have already been selected during evolution. Interactions between residues close in the tertiary structure may be important, but such interacting residues may be far apart in primary structure, limiting in vitro combinatorial mutagenesis. Furthermore, deletions, insertions, or associations between subunits are difficult to manipulate by random genetic-selection strategies, even though such "irreversible" changes during evolution may account for major limitations on Rubisco catalytic efficiency.

Schemes for genetic selection are further confounded by the diversity of cellular environments. For example, Rubisco enzymes from photosynthetic microorganisms that contain CO_2-concentrating mechanisms generally have higher K_c values than those of Rubisco enzymes from C_3 plants that lack such concentrating mechanisms (57). One might succeed in selecting a better enzyme by growing these organisms under CO_2-limited conditions (131) only to find that a lower K_c or higher K_o/K_c, which is often obtained at the expense of a decrease in V_c (reviewed in 125), provides no benefit because the CO_2-concentrating mechanism provides sufficient CO_2 to saturate the enzyme. One can, of course, argue indefinitely about the utility of selection strategies, but no Rubisco enzyme has yet been selected that improves Pn in vivo except for normal-Ω revertants of low-Ω Rubisco mutants of *Chlamydomonas* (reviewed in 125).

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DISTANT EFFECTS AND RATIONALE FOR IMPROVEMENT The most obvious targets for improving Rubisco are the 20 residues in van der Waals contact with CABP in the Rubisco active site (3). Directed mutagenesis of these "active-site" residues in *R. rubrum*, *Synechococcus*, *Chlamydomonas*, or tobacco Rubisco has shown that all are required for maximal rates of catalysis (19, 153; reviewed in 53, 124, 125). Furthermore, these active-site residues cannot account for the variation in kinetic parameters observed between Rubisco enzymes from different species (57, 102, 139, 151) because they are nearly 100% conserved among thousands of large-subunit sequences. Thus, analysis of active-site residues is important for understanding the details of the Rubisco catalytic mechanisms, but these highly conserved and essential residues might not be the best targets for engineering a better enzyme. A more promising strategy for the eventual design of an improved enzyme is to examine variable residues and regions farther from the active site.

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INTERACTIONS IN THE RUBISCO LARGE SUBUNIT

Whereas all Rubisco X-ray crystal structures show very similar Cα backbone structures, there is substantial divergence in amino-acid side chains. Thus, it is difficult to correlate differences in structure with differences in kinetic properties. It has also been a challenge to apply genetic methods for investigating divergent Rubisco enzymes. Because eukaryotic Rubisco holoenzymes fail to assemble when their subunits are expressed in *Escherichia coli* (24, 42), most directed mutations have been made in the *R. rubrum* or *Synechococcus* Rubisco enzymes expressed in *E. coli* (reviewed in 53, 124). Only in tobacco and the green alga *Chlamydomonas reinhardtii* has it become possible to transform the chloroplast genome as a means for analyzing the effects of mutant large subunits on the function of eukaryotic Rubisco (153, 158, 159). Although progress has been made in eliminating or substituting *rbcL* genes in photosynthetic prokaryotes (38, 96, 99), these systems have yet to be exploited for the genetic dissection of Rubisco structure/function relationships (reviewed in 124, 132).

Mutational Approaches

The first attempt to step outside the sphere of the active-site residues was made using classical genetics with *Chlamydomonas* (reviewed in 125). Because this organism can survive in the absence of photosynthesis when supplied with acetate as a source of carbon and energy and continues to synthesize a complete photosynthetic apparatus even when grown in darkness, a number of *rbcL* missense mutants were recovered by screening acetate-requiring strains (G54D, G171D, T173I, R217S, G237S, L290F, V331A) (reviewed in 124, 125). Four of the missense mutants (G54D, R217S, L290F, V331A) and their suppressors have defined regions relatively far from the active site that can influence Ω. These regions include the secondary structural elements close to the loops that contain Lys-201 and Lys-334 (14, 17, 137), as well as regions buried within the N-terminal domain

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and at the interface between large and small subunits (13, 31, 54, 128). Despite the fact that Rubisco is required for the survival of land plants, two *rbcL* missense mutations (S112F and G322S) that disrupt holoenzyme assembly have been identified in variegated mutants of tobacco (8, 121). Missense mutations recovered by screening are not distributed randomly within the gene because only those that affect essential structural or functional properties will be observed (124). Thus, most of the resulting amino-acid substitutions are likely to provide useful information about catalysis or assembly. For reasons that are not readily apparent, no *rbcL* structural-gene mutation has yet been described from the screening of photosynthesis-deficient prokaryotes (2, 132).

Scanning mutagenesis, in which all residues of a certain type are replaced by directed mutagenesis, has been used to examine those Gly residues that are conserved among all Rubisco large subunits (20, 71). When each of the 22 Gly residues in the *Synechococcus* enzyme was replaced with either Ala or Pro, only the G47A, G47P, G122A, G171A, G179A, G403A, G405A, and G416A enzymes retained some carboxylase activity. However, in the absence of detailed kinetic analysis, it is not known whether further study of the regions affected by these substitutions would be informative. Other conserved large-subunit residues distant from the active site have been replaced by directed mutagenesis with effects on Rubisco function or stability (9, 31, 88; reviewed in 124). For example, when Cys-172 was replaced with Ser in the *Chlamydomonas* large subunit, an increase in holoenzyme stability in vivo was observed under oxidative stress conditions that triggered holoenzyme degradation (88). However, as noted above, replacement of conserved residues is not expected to account for differences in catalysis among divergent enzymes.

Most directed mutagenesis studies aimed at examining distant interactions have relied on a phylogenetic approach in which the identities of residues are changed from those of one species to those of another species of Rubisco. The few conserved differences in sequences between C_3 and C_4 plant Rubisco and between Rubisco enzymes that display different specificities for interaction with Rubisco activase have been identified. However, because a variety of land-plant Rubisco enzymes cannot be genetically engineered, conclusions must be based primarily on alterations of the *Synechococcus* or *Chlamydomonas* enzyme (86, 93). The major limitation to the phylogenetic approach is, once again, deciding which of the many divergent regions are worth analyzing (e.g., 86, 93, 140). Nonetheless, by combining this approach with others, two large-subunit regions have been investigated intensely. One of these regions is close to the active site, and the other is distant.

Loop-6 Amino-Acid Substitutions

Prior to solving the X-ray crystal structure of the dynamic loop 6 (68), mutant screening and selection in *Chlamydomonas* revealed that a V331A substitution in this loop caused decreases in V_c and Ω (14). The second-site suppressor substitutions T342I and G344S in α-helix 6 complemented the V331A substitution

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and increased V_c and Ω to levels sufficient for restoring photoautotrophic growth to the mutant cells (14, 17). When the T342I substitution was created alone in *Synechococcus* Rubisco, little or no change occurred in Ω, but V_c was decreased and K_m (RuBP) was increased (46, 107). Whereas T342I appears to complement V331A by replacing bulk in the hydrophobic core of the loop (14, 68), the recently solved crystal structure of *Chlamydomonas* Rubisco (135a) confirmed that the G344S substitution may complement V331A by a different mechanism (Figure 2*A*). Nonetheless, changes in these residues likely affect the discrimination between CO_2 and O_2 by altering the placement of active-site Lys-334 (15, 49) (Figure 2). Deletion of loop-6 residues eliminates function (27, 72), and every conserved loop-6 residue that has been substituted causes a dramatic decline in V_c or Ω (where measured) (46, 75, 107, 153; reviewed in 124, 125). However, engineered substitutions at the conserved, N-terminal-domain Lys-128, which may hydrogen bond with the carboxyl group of Val-331 (Figure 2), also causes decreases in V_c and Ω (9), indicating that changes in loop 6 might influence catalysis by altering the orientation of other active-site residues like Asn-123 (18, 122, 158).

Taking a phylogenetic approach, several investigators have changed the *Synechococcus* α-helix 6 sequence DKAS (residues 338–341) to the EREI or ERDI sequence characteristic of land plants (Figure 2, compare panels *B* and *C*) (46, 61, 94). In all of these studies, V_c was decreased by 8–40%. The lower V_c is characteristic of land-plant Rubisco, but the Ω value did not increase to that of a land-plant enzyme. Similarly, single substitutions in loop 6 of the *Synechococcus* enzyme had either minimal or negative influence on the catalytic properties of Rubisco (81, 94).

Following the discovery that Rubisco enzymes of nongreen, eukaryotic algae generally have higher Ω values than those of land-plant enzymes (101, 102, 139) and are quite divergent in loop-6 sequences (Figure 2), the *Synechococcus* sequence KASTL (residues 339–343) was changed to either the PLMIK or the PLMVK sequence characteristic of *Cylindrotheca* or related nongreen algae (100, 107). However, these mutations reduced both V_c and Ω, and a variety of single residue substitutions produced similar results (100, 107). The *Synechococcus* sequences VDL (residues 346–348) was also changed to YNT or YHT, but these substitutions blocked the assembly of a functional holoenzyme in *E. coli* (100, 107). Loop-6 residues that differ between two species are likely to be complemented by additional residues outside of this loop that also differ (Figure 2). However, to examine only eight residues that differ between two enzymes (in all single, pairwise, and multiple combinations) would require the daunting task of creating and analyzing 256 mutant enzymes!

Only two studies have examined divergent residues involving loop 6 that are far apart in primary structure but close together in tertiary structure (46, 159). Because the C-terminal end of the large subunit interacts with loop 6 (Figure 2), a *Synechococcus* mutant enzyme was created in which both the ETMDKL C-terminal end and the DKAS loop-6 residues (nos. 338–341) were changed to those of spinach Rubisco (PAMDTV and ERDI, respectively). However, the properties of

this mutant enzyme were not different from the properties of the mutant enzymes in which each of the two regions was changed separately (i.e., 30% decrease in V_c but no change in Ω) (46).

In contrast to *Synechococcus*, there are only three residues in loop 6 that differ between *Chlamydomonas* (Leu-326, Val-341, Met-349) and land-plant (Ile-326, Ile-341, Leu-349) Rubisco. Whereas a V341I substitution in the *Chlamydomonas* enzyme had little effect, an L326I substitution decreased holoenzyme stability in vitro and in vivo (159). This decrease in stability could be partially complemented by an M349L substitution on the opposite side of the loop (Figure 2, compare panels *A* and *B*). Thus, the divergent pair of residues in van der Waals contact at the base of loop 6 may be retained during evolution owing to its contribution to holoenzyme stability. However, the L326I/M349L double-mutant enzyme had substantial decreases in V_c and Ω (159). The catalytic efficiency and specificity of land-plant Rubisco must be maintained by other residues that complement Ile-326 and Leu-349, and these residues must be different from those that complement the analogous loop-6 residues in *Chlamydomonas*. Because only four of the residues that interact with this region of loop 6 differ between *Chlamydomonas* and spinach (Figure 2, compare panels *A* and *B*), it should be possible to create and analyze a complete set of mutant enzymes.

Bottom of the Barrel

By mutant screening and selection in *Chlamydomonas*, an L290F substitution at the bottom of β-strand 5 was found to decrease V_c and Ω (13), and A222T (in α-helix 2) and V262L (below β-strand 4) suppressor substitutions were recovered that restored Ω to the wild-type level (54). The L290F mutant is a temperature-conditional, acetate-requiring strain. Its mutant Rubisco supports photoautotrophic growth at 25°C, but the enzyme is unstable and degraded at 35°C in vivo (13). The A222T and V262L substitutions not only improve the catalytic efficiency of L290F, but also its thermal stability in vivo and in vitro (31, 54). When present alone, the A222T and V262L suppressor substitutions each improved the thermal stability of otherwise wild-type Rubisco in vitro with only a slight decrease in V_c (31). Although A222T and V262L are improved enzymes with respect to thermal stability, *Chlamydomonas* does not live at temperatures where this enhanced thermal stability can be manifested (60°C). Nonetheless, these results indicate that it may be possible to select for improved Rubisco under conditions not previously encountered by the organism during evolution.

The "long distance" interactions between residue 290 and residues 222 and 262 are particularly interesting because all three residues are in contact with the βA-βB loop of the small subunit (Figure 3*A*). The importance of this small-large-subunit interface, which is approximately 20 Å away from the active site, was recently confirmed when small-subunit N54S and A57V suppressor substitutions were found that restored Ω and thermal stability of the L290F enzyme back to wild-type values (Figure 3*A*) (30). All of these substitutions may affect a hydrogen-bond network (68) that extends from the region of Leu-290 and culminates at active-site

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residue His-327 (30, 31, 54). Nonetheless, subtle changes quite far from the active site can influence catalytic efficiency, indicating that small-subunit residues may also be appropriate targets for genetic engineering of an improved Rubisco.

Only three large-subunit residues (nos. 256, 258, 265) differ between *Chlamydomonas* and spinach Rubisco in the region surrounding the small-subunit βA-βB loop (Figure 3, compare panels *A* and *B*). In a recent study (Y. C. Du & R. J. Spreitzer, unpublished), C256F, K258R, and I265V substitutions were created by directed mutagenesis and chloroplast transformation, thereby converting *Chlamydomonas* residues to the corresponding residues of spinach Rubisco (Figure 3, panels *A* and *B*). Whereas the single- and double-mutant substitutions have only minor effects on catalysis, Rubisco from the C256F/K258R/I265V triple-mutant strain, which can survive photoautotrophically, has a 10% decrease in Ω, largely owing to a substantial decline in V_c. Once again, there must be different residues in spinach Rubisco that complement Phe-256, Arg-258, and Val-265. However, these divergent residues may reside within the closely associated small subunit (Figure 3).

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INTERACTIONS INVOLVING THE SMALL SUBUNIT

The existence of Form II Rubisco enzymes, composed of only large subunits, indicates that small subunits are not absolutely essential for carboxylase activity. Furthermore, *Synechococcus* large-subunit octamers (void of small subunits) retain ~1% carboxylase activity and have a normal Ω value (5, 44, 74, 87). However, V_c is drastically reduced in such minimal enzymes, and a variety of side products are produced by misprotonation of RuBP (87). Scanning mutagenesis of cyanobacterial small subunits, expressed with large subunits in *E. coli*, has also shown that substitutions at some of the conserved residues can decrease V_c and holoenzyme assembly (41, 69; reviewed in 124, 126). Although it is apparent that small subunits can influence catalysis indirectly, it has been difficult to determine whether divergent small-subunit residues play a role in the differences in kinetic constants among Rubisco enzymes from different species. *R. rubrum* Rubisco lacks small subunits, small subunits of nongreen algae are encoded by the chloroplast genome (which has yet to be transformed), and land plants have a family of *rbcS* genes in the nucleus that cannot be readily eliminated (43, 65, 152; reviewed in 124, 126).

Hybrid Holoenzymes

Because small-subunit primary structures are more divergent than large-subunit sequences, it is reasonable to consider whether small subunits contribute to the phylogenetic differences in Rubisco catalytic efficiency and Ω. When large subunits from *Synechococcus* ($\Omega = 40$) were assembled with small subunits from spinach ($\Omega = 80$) or *Alcaligenes eutrophus* ($\Omega = 74$), the hybrid enzymes had Ω values comparable to that of the *Synechococcus* holoenzyme despite substantial decreases in V_c (7, 74). In contrast, coexpression of large subunits from *Synechococcus*

($\Omega = 40$) and small subunits from the diatom *Cylindrotheca* ($\Omega = 107$) in *E. coli* produced a hybrid holoenzyme that had an intermediate Ω value of 65 (101). By exploiting chloroplast transformation of tobacco, large subunits from sunflower ($\Omega = 98$) were assembled with the resident small subunits of tobacco ($\Omega = 85$) to produce a hybrid holoenzyme that also had an intermediate Ω value of 89 (60). Because the Ω values of hybrid Rubisco enzymes can, in some cases, be influenced by the contributed small subunits, one might hope to improve Rubisco by providing foreign small subunits. However, the resulting hybrid holoenzymes had greater than 80% decreases in V_c (60, 101). Furthermore, because there are substantial differences in small-subunit sequences (29 residues differ between tobacco and sunflower), it is difficult to determine which part(s) of the small subunit may contribute to differences in catalytic efficiency and Ω.

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Small-Subunit βA-βB Loop

The small subunits of prokaryotic and nongreen-algal Rubisco lack 10 residues of a 22-residue loop between β strands A and B that is characteristic of land-plant small subunits (Figure 3). This βA-βB loop contains 27 residues in the small subunits of green algae (Figure 3*A*). In land plants and green algae, the loop extends between and over the ends of two large subunits from the bottom side of the α/β barrel and interacts with large-subunit α-helices 2 and 8, as well as with the βA-βB loops of two neighboring small subunits (3, 68, 135a). The long C-terminal extensions of some prokaryotic and nongreen-algal small subunits form β loops that place residues into positions similar to the residues in the βA-βB loops of plants and green algae (47, 130) (Figure 3, compare panel *D* with panels *A* and *B*). Because the βA-βB loop region is the most divergent structural feature of Rubisco enzymes, it may account for differences in catalytic efficiency and Ω.

To examine the significance of these residues, the *Synechococcus* βA-βB loop was replaced with the βA-βB loop of the pea small subunit (148). Whereas the wild-type *Synechococcus* small subunit could not assemble with pea large subunits in isolated pea chloroplasts, the chimeric small subunit was now able to assemble owing to the presence of the pea βA-βB loop. A number of amino-acid substitutions were created within the pea βA-βB loop (R53E, E54R, H55A, P59A, D63G, D63L, Y66A), but only R53E blocked holoenzyme assembly (1, 40). Because these studies relied on import of small-subunit precursors into isolated chloroplasts, insufficient amounts of Rubisco could be isolated to determine the influence of the βA-βB-loop substitutions on Rubisco catalysis.

Because N54S and A57V suppressor substitutions in the βA-βB loop of the *Chlamydomonas* small subunit could increase the V_c, Ω, and thermal stability of the large-subunit L290F mutant enzyme (30) (Figure 3*A*), it seemed likely that the βA-βB loop might also have a direct influence over Rubisco catalysis. Using a mutant of *Chlamydomonas* that lacks the *rbcS* gene family as a host for transformation (65), five βA-βB-loop residues conserved in sequence between *Chlamydomonas* and land plants, but different or absent from the corresponding loops of prokaryotes and nongreen algae, were each replaced with Ala (i.e., *Chlamydomonas*

Arg-59, Tyr-67, Tyr-68, Asp-69, Arg-71) (Figure 3*A*) (127). Although none of the substitutions eliminated holoenzyme assembly, most of the mutant enzymes had decreased V_c values, and the R71A enzyme had a reduction in Ω (127). Because Arg-71 can influence Ω and differs relative to the analogous residues of *Synechococcus* (Phe-53) and *Galdieria* (Ala-46) (Figure 3), this residue, and those with which it interacts, may contribute to the differences in catalytic properties between divergent Rubisco enzymes. Thus, the small subunit, and the βA-βB loop in particular, may also be a suitable target for future attempts at engineering an improved Rubisco.

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REGULATORY INTERACTIONS OF RUBISCO ACTIVASE

The active site of Rubisco assumes a closed conformation with certain phosphorylated ligands regardless of the carbamylation state of Lys-201 (3, 133–136). This finding is consistent with kinetic evidence indicating that RuBP and its epimer, xylulose bisphosphate, bind very tightly to uncarbamylated sites, and even tighter ($\sim 10^3$ times) than to sites that are carbamylated (56, 157). Duff et al. (32) have proposed that a 9.2-Å distance between the P1 and P2 phosphates of a bisphosphorylated compound is required for the closed conformation. The crystal structure of Rubisco with its carboxylation product, 3-phosphoglyceric acid, is in an open conformation (134), indicating that C─C bond cleavage of the carboxylated C_6 or oxygenated C_5 intermediate during normal catalysis is apparently sufficient to open a closed active site (6, 32). The very tight binding of RuBP to uncarbamylated sites indicates that, once closed, these sites are very slow to open. Thus, the closed conformation represents a potential dead end for uncarbamylated sites because these sites are unable to trigger opening via C─C bond cleavage. That binding of RuBP to the uncarbamylated, low-Ω Rubisco of photosynthetic bacteria is much less tight (56) indicates that tight binding of RuBP to uncarbamylated sites may have developed during evolution as a consequence of increased specificity for CO_2.

Opening the Closed Active Site

In the absence of catalysis, conversion of Rubisco from the closed to the open conformation is extremely slow. To facilitate the process, plants contain Rubisco activase, an ATP-dependent enzyme that releases tight-binding sugar phosphates from the Rubisco active site (reviewed in 97, 113). Activase is an AAA^+ protein, a member of a superset of proteins related to the AAA (ATPases associated with a variety of cellular activities) family that includes a wide variety of proteins with chaperone-like functions (90). Common to each is a core AAA^+ module that contains 11 motifs, some of which, like the P-loop and Walker A and B sequences, are highly conserved among ATPases (90).

Activase interacts with Rubisco, somehow facilitating the release of bound sugar phosphates from the active site (145). The interaction almost certainly involves changing the conformation of Rubisco in a way that promotes opening of the closed configuration. Once activase opens a closed site, the sugar phosphate can dissociate

and free the site for activation via spontaneous carbamylation and metal binding (145, 149). Studies with the activase-minus *Arabidopsis rca* mutant (115) and antisense tobacco and *Arabidopsis* plants (33, 84) have shown that photosynthesis at atmospheric levels of CO_2 is severely impaired when plants lack activase because Rubisco becomes sequestered in an inactive form. Thus, activase would be required in all photosynthetic organisms that contain a Rubisco whose active sites are prone to forming a tightly closed conformation with RuBP in the uncarbamylated state.

Activase protein has been detected in all plant species examined, including both C_3 and C_4 plants and green algae (116). GenBank contains entries for activase gene sequences from at least 21 different land-plant species and three species of green algae. An activase-like gene has also been identified in the cyanobacterium *Anabaena* (76). The recombinant product of this activase-like gene catalyzed ATP hydrolysis but was not functional in relieving inhibition of Rubisco by carboxyarabinitol 1-phosphate (77).

Because of the dependence on ATP hydrolysis (105, 129) and, thus, stromal ATP, the controlled switching of Rubisco active sites from the closed to open conformation by activase forms the basis for the regulation of Rubisco by light (97, 113). Activase also facilitates the release of compounds that induce the closure of loop 6 upon binding to carbamylated sites (78, 104, 106, 145). Thus, activase is also required in organisms that contain certain tight-binding inhibitors that sequester carbamylated active sites in the closed conformation. The occurrence, synthesis, and properties of these compounds, which include 3-ketoarabinitol bisphosphate, a compound produced at the active site by misprotonation of RuBP, and carboxyarabinitol 1-phosphate, a naturally occurring inhibitor in some plants, have been discussed in detail by others (6, 34, 64, 119, 157).

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Structure/Function Relationships of Activase

ACTIVASE STRUCTURE For many molecular chaperones, the AAA^+ module is linked covalently to domains that determine the actual cellular function (90). The ATPase activity of the AAA^+ module acts as a motor driving the intermolecular interactions that are required for function. The active molecule is usually multimeric, composed of many AAA^+ subunits often assembled in rings (90 and references therein). In the case of activase, the active form appears to be an oligomer of 14 or perhaps 16 subunits (78, 146).

The activase subunits are highly self-associating, increasing in activity with the extent of oligomerization (111, 146). Oligomerization occurs in response to the binding of ATP or its nonhydrolyzable analog ATPγS. However, ATPγS does not substitute for ATP in Rubisco activation, indicating that oligomerization of activase occurs first, followed by hydrolysis of ATP either before or during the interaction with Rubisco to drive conformational changes in the latter. Mixing experiments with mutant and wild-type activases expressed in *E. coli* have also shown that the subunits function cooperatively (111, 142), consistent with the presence of interactive domains on adjacent subunits, a common feature in AAA^+

proteins (90). The consensus AAA^+ module of activase is most similar in sequence to the cell-division-cycle protein 48 and the ATP-dependent regulatory subunit of the eukaryotic 26S protease, a member of the Clp/Hsp100 family. Another member of this family, Hsp104, exhibits ATP- and protein-concentration-dependent association and subunit cooperativity very reminiscent of activase (117). Preliminary analysis of the activase-Rubisco complex by electron microscopy indicates that activase subunits may encircle Rubisco (11), similar to the interaction of the GroEL-type chaperonins with unfolded proteins, including the Form II Rubisco subunits (143).

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INTERACTIONS WITH RUBISCO Several studies have attempted to define the regions of activase necessary for its interaction with Rubisco. Taking advantage of the species specificity of activase (see below), Esau et al. (36) used chimeric activase proteins to show that the C-terminus of activase is important for recognizing Rubisco. Studies with mutants truncated at the N-terminus have shown that the first 50 amino acids of the mature activase protein are not required for ATPase activity but are required for Rubisco activation (141). Within the N-terminus of activase, a conserved Trp residue at position 16 appears to be involved in the interaction between Rubisco and activase (37, 141). Thus, both the C- and N-terminal regions of activase, which lie outside the AAA^+ module, appear to be involved in the interaction with Rubisco.

Opening of the closed conformation of Rubisco may involve a physical interaction between activase and Rubisco. Although no stable complex has been isolated, evidence from cross-linking studies (154), immunoprecipitation (154, 156), and electron microscopy (11) favor the possibility of a direct interaction. The most compelling evidence is the species specificity exhibited by activase from members of the Solanaceae like tobacco (147). Activase from tobacco is an inefficient activator of Rubisco from non-Solanaceae land plants and the green-alga *Chlamydomonas* but is active toward tobacco Rubisco and Rubisco from two other Solanaceae plants. The opposite result was obtained with spinach activase. P89R and D94K amino-acid substitutions, introduced into the large subunit of *Chlamydomonas* Rubisco to change specific surface residues to those characteristic of Solanaceae Rubisco (97), altered the species specificity of activase (73, 93). The results suggest the possibility of an activase-recognition region formed, in part, by the loop between β-strands C and D on the surface of the large-subunit N-terminal domain (Figure 4).

The mechanism by which activase alters the active site of Rubisco from a closed to an open conformation is unknown. The process is coupled to ATP hydrolysis, either for priming activase for its interaction or during the actual conformation-changing event. Duff et al. (32) have suggested that the activase-Rubisco interaction may involve Asp-473, the latch site that they proposed stabilizes the closed conformation of Rubisco (Figure 4). The C-terminus of the large subunit also contains two residues that differ between Solanaceae and non-Solanaceae enzymes, and these residues reside near the surface of the N-terminal domain of a neighboring

large subunit when Rubisco is in the closed conformation (Figure 4, compare panels *A* and *B*).

ACTIVASE ISOFORMS An interesting but complicating feature of activase is the presence of two subunits of approximately 47 kDa and 42 kDa in many plant species (116). In all cases that have been reported to date, alternative splicing of a pre-mRNA produces these two proteins that are identical except for the presence of an extra 27–36 amino acids at the C-terminus of the longer form (109, 138, 150). The two activase polypeptides are active both in ATP hydrolysis and Rubisco activation (120), but they differ in kinetic properties (26, 120, 155), as well as in their thermal stabilities (26). Zhang & Portis (155) have shown that the longer subunit type is subject to redox regulation via thioredoxin-f-mediated reduction of a pair of Cys residues in the C-terminal extension. Reduction decreases the sensitivity of activase to inhibition by ADP, both the longer form per se and heteromers containing both forms of activase. Redox regulation of activase would serve a regulatory role, adjusting activase activity to light intensity by fine tuning the sensitivity of the enzyme to inhibition by ADP.

Alternative splicing is usually responsible for production of the two forms of activase. However, the two forms are encoded by different activase genes in cotton (M. E. Salvucci, unpublished). Another exception is barley, which has two activase genes, only one of which is alternatively spliced (109). In several plant species, including tobacco, tomato, maize, and *Chlamydomonas*, only a single activase subunit type is produced under nonstress conditions (67, 103, 116). Although these species contain the shorter form of activase that does not harbor the two Cys residues necessary for redox regulation, irradiance levels still affect the activation state of Rubisco in these plants in much the same way as in plants that contain both forms of activase (112). Thus, questions remain concerning activase and Rubisco regulation in plants containing only the shorter form of activase and the occurrence and function of multiple activase genes.

Activase as a Potential Target for Increasing Photosynthesis

Because most strategies for improving Rubisco require a change in the structure of the enzyme, it is necessary to consider how each change will affect the interaction of Rubisco with activase, chaperonins, and other proteins necessary for assembly or function (reviewed in 126). For example, improvements that involve replacement of Rubisco subunits, even if properly assembled, could be ineffective in vivo if activase is unable to recognize the Rubisco and reverse formation of dead-end complexes (60). This problem might even occur with single amino-acid substitutions if they alter recognition of Rubisco by activase (73, 93). Thus, each strategy for improving Rubisco should be mindful of the possible need to co-design activase. Redesigning activase will require a more complete understanding of the mechanism of action and the sites for interaction with Rubisco.

Because the activation state of Rubisco limits photosynthesis under conditions of high CO_2 and temperature, improvements in activase may stimulate photosynthesis under certain conditions (25). For example, the decrease in Rubisco

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activation that occurs in response to elevated CO_2 appears to involve limitation of activase by [ATP] (25, 110). Engineering activase to be less sensitive to inhibition by ADP, either by directed mutagenesis (59) or by altering the relative expression of the two forms (26, 155), may improve the performance of plants under high CO_2. At elevated temperatures, Rubisco activation decreases to levels that limit photosynthesis because activase is unable to keep pace with the much faster rate of Rubisco deactivation (25, 39). The poor performance of activase at high temperature is caused, in part, by its exceptional thermal lability (114). Thus, changes in activase that improve its thermal stability or increase its amount represent possible approaches for increasing photosynthesis at elevated temperatures. The latter approach may be especially useful in C_4 plants, which have elevated levels of CO_2 at the site of Rubisco but do not exhibit a marked stimulation of photosynthesis by temperature because of lower Rubisco activation (S. J. Crafts-Brandner & M. E. Salvucci, unpublished).

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PROSPECTS FOR IMPROVEMENT

Gross changes in the gaseous composition of the earth's atmosphere has selected for land-plant Rubisco with a relatively high Ω. By comparison, the evolutionarily pressure to optimize the land-plant enzyme for performance in controlled agricultural settings has been minimal and indirect. Instead, land plants have evolved under natural conditions where the availability of water and/or nitrogen often limits photosynthesis. As shown by the many studies involving CO_2-enrichment, increasing the rate of carboxylation by Rubisco will increase plant yield, provided that sufficient nitrogen is available for increased protein synthesis. Improving the catalytic efficiency of Rubisco will have the same effect but require less nitrogen to implement.

As the new century opens, the prospects for improving Rubisco are excellent. Elucidation of the X-ray crystal structures of Rubisco in its many forms has provided a structural framework for understanding Rubisco function and evaluating the effects of mutations (3, 32, 91, 135a). Coupled with thousands of Rubisco sequences, these structures may guide phylogenetic and bioinformatic inquiries into the diversity of kinetic parameters. Successful efforts to produce mutations in the chloroplast-encoded large subunit (153, 158) and to replace subunits (60, 65, 127) have laid the groundwork for applying new approaches to understanding Rubisco structure/function relationships. Finally, the realization that Rubisco function is tied to and can be limited by its interaction with activase (25) indicates that improvements in activase may provide a totally new approach for enhancing photosynthesis.

ACKNOWLEDGMENTS

We thank Vijay Chandrasekaran, Patrick D. McLaughlin, and Sriram Satagopan for preparing the figures and acknowledge research support from the U.S. Department of Agriculture (and its National Research Initiative) and Department of Energy.

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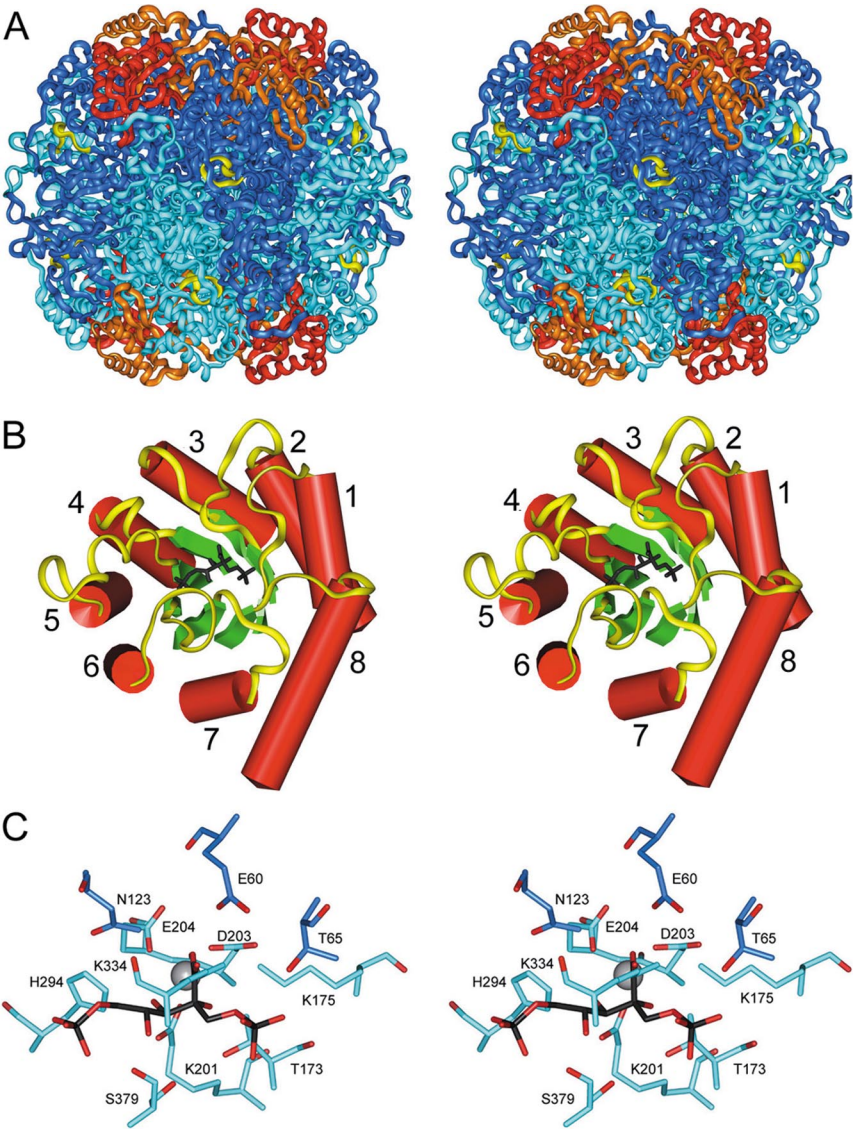

Figure 1 Stereo images of the X-ray crystal structure of spinach Rubisco (8RUC) in relation to catalysis (3). (*A*) The holoenzyme is composed of eight large subunits (*dark blue*, *light blue*) and eight small subunits (*red*, *orange*). Active sites that form between two neighboring large subunits are denoted by loop 6 (*yellow*). (*B*) The C-terminal domain of each large subunit forms an α/β-barrel. Loops (*yellow*) between β strands (*green*) and α helices (*red*) contain residues that interact with the transition-state analog CABP (*black*). (*C*) C-terminal-domain residues (*light blue*) from one large subunit and N-terminal-domain residues (*dark blue*) from a neighboring large subunit interact with CABP (*black*). Mg^{2+} is denoted as a *gray sphere*. Oxygen atoms are colored *red*.

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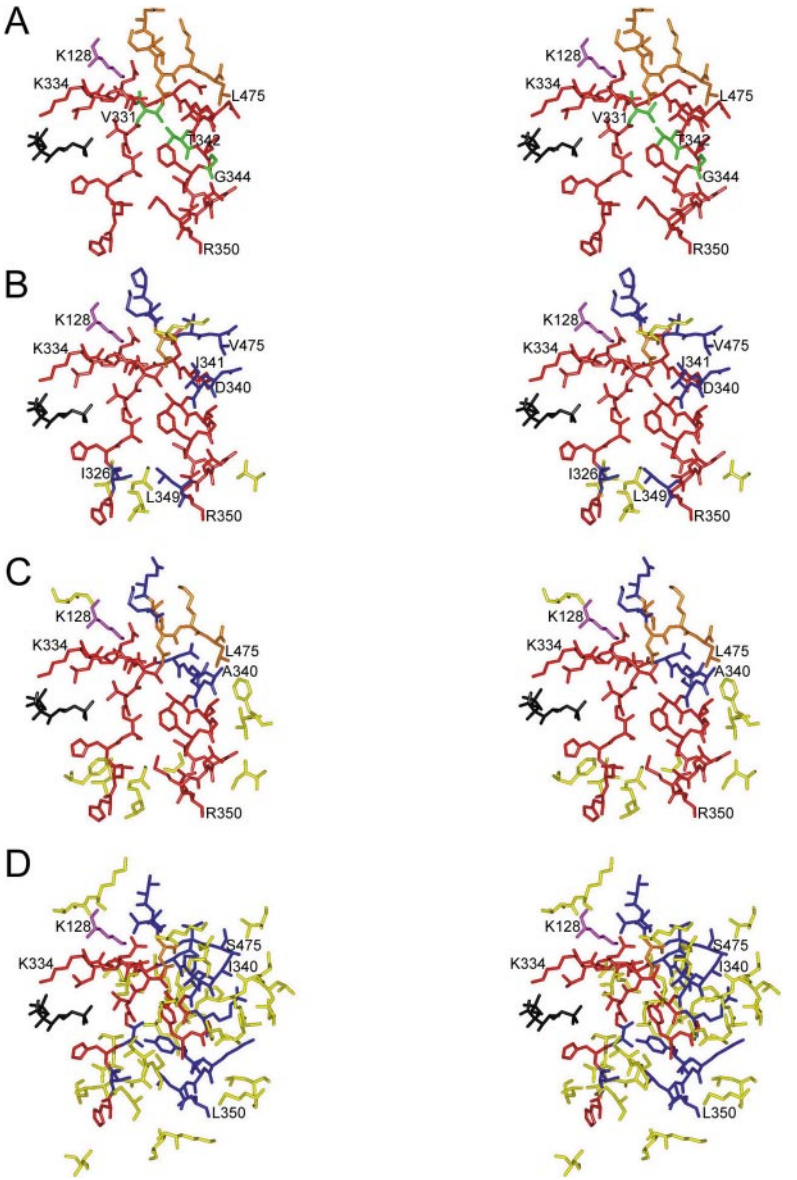

Figure 2 Stereo images of the loop-6 region of Rubisco from (*A*) *Chlamydomonas reinhardtii* at 1.4-Å resolution (135a), (*B*) spinach (8RUC) (3), (*C*) *Synechococcus* (1RBL) (91), and (*D*) *Galdieria* (1BWV) (130). Loop-6 residues (*red*) affected by complementing mutant substitutions in *Chlamydomonas* are colored *green* (17). Loop-6 residues and C-terminal residues (*orange*) that differ relative to those of *Chlamydomonas* are colored *blue*. Other residues that differ from those of *Chlamydomonas* within 5 Å of the loop-6 or C-terminal residues are colored *yellow*. CABP is colored *black*.

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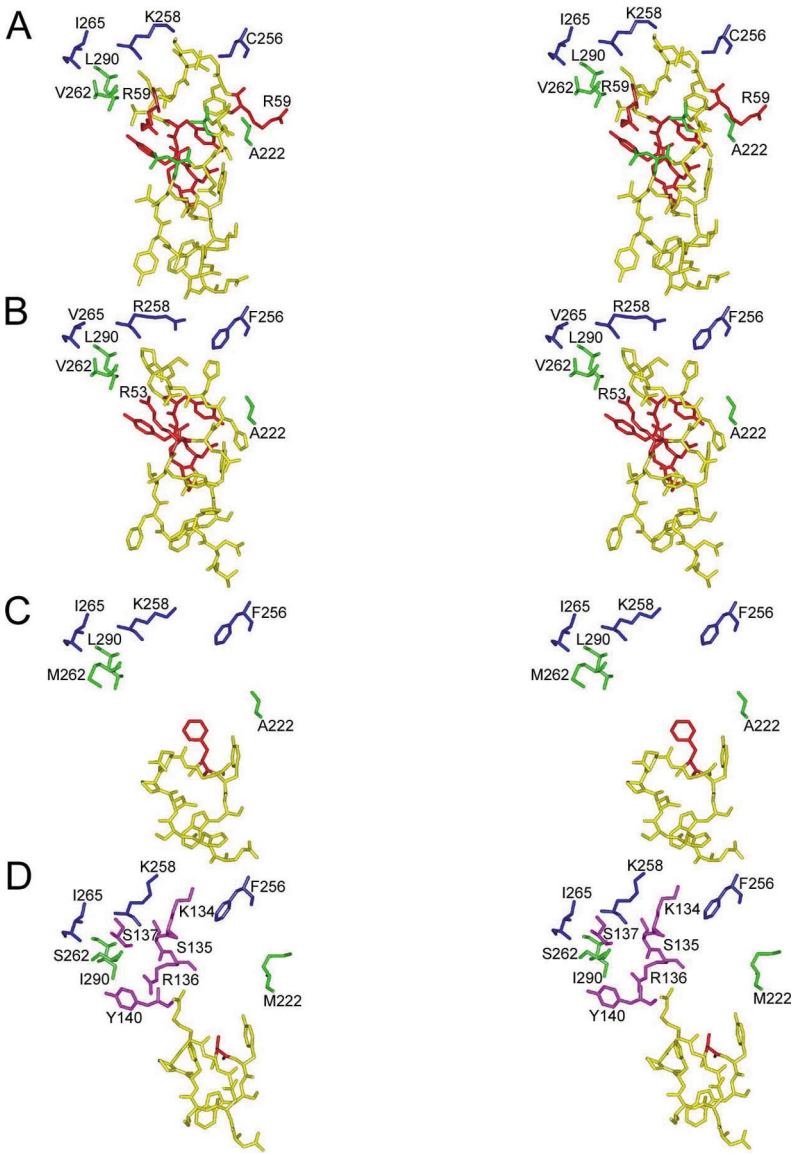

Figure 3 Stereo images of pertinent large-subunit residues (residues 219–290) that flank the small-subunit βA-βB loop (*yellow*) of (*A*) *Chlamydomonas* at 1.4-Å resolution (135a), (*B*) spinach (8RUC) (3), (*C*) *Synechococcus* (1RBL) (91), and (*D*) *Galdieria* (1BWV) (130). Conserved and analogous residues within the small-subunit βA-βB loops are colored *red*. Arg-59 from a neighboring *Chlamydomonas* small subunit is colored *orange*. Residues altered by screening and selection in *Chlamydomonas* are colored *green* (13, 30, 54). *Galdieria* C-terminal small-subunit residues are colored *violet*.

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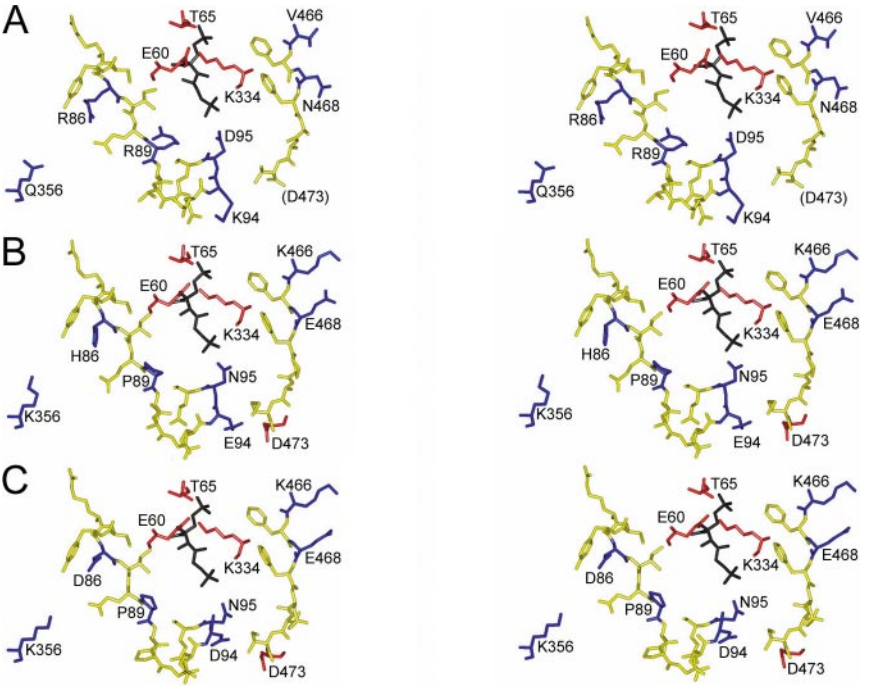

Figure 4 Stereo images of Rubisco large-subunit residues that may account for the species specificity of interaction with Rubisco activase (147). (*A*) tobacco (4RUB) (118), (*B*) spinach (8RUC) (3), and (*C*) *Chlamydomonas* at 1.4-Å resolution (135a). Surface residues that differ in charge between Solanaceae (tobacco) and non-Solanaceae (spinach and *Chlamydomonas*) species are in *blue* (73, 93). Pertinent active-site residues and the Asp-473 "latch" residue (32) (which is not visible in the tobacco structure) are colored *red*. CABP is colored *black*.

Annual Review of Plant Biology
Volume 53, 2002

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